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14. ABSTRACT

The purpose of this project is to develop and test a system that identifies in real time the effects of breast cancer cells on gene transcription by cells in their microenvironment. The accomplishment of this goal will identify messages induced in normal cells adjacent to cancer cells that support cancer cell growth and survival. In this Concept award project, we are testing whether breast cancer cells that make lysyl oxidase (a metastasis promoting enzyme) or have lysyl oxidase suppressed differ in their ability to turn on hospitable genes in bone marrow stromal cells. We have successfully tagged bone marrow stromal cells with a protozoal enzyme (uracil phosphoribosyl transferase) that enables us to measure ongoing stromal cell transcription when stroma is mixed with breast cancer cells. Real time transcription specific to the stromal cell compartment was able to be measured without disaggregating stromal and cancer cells, so that genetic signals arising from cell cell contact between stroma and cancer cellscould be captured. A global assessment of stromal genes induced by high versus low metastatic cancer cells is planned. This project is likely to help guide the development of stromal-based therapeutics.

15. SUBJECT TERMS
microenvironment, bone marrow metastases, breast cancer, transcription, uracil

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INTRODUCTION

This proposal establishes a new paradigm for detecting the transcriptional activities specific to stromal cells that are in contact with breast cancer cells without disaggregating the stromal and cancer cells. This approach is used to test the hypothesis that lysyl oxidase (LOX) increases breast cancer metastasis (1) in part by altering gene transcription in cells adjacent to the cancer cells. Despite the importance of cancer cell-stromal interactions (2), current approaches are poorly suited to analyze the effect of cancer cells on their microenvironment (and vice versa) within intact tumors. The proposed investigations overcome these limitations through use of a toxoplasma ghondii enzyme. It should then be feasible for the first time to kinetically profile transcription that arises from cell-cell contacts in distinct components of the cancer microenvironment. This proof-of-principle investigation determines how gene transcription in bone marrow stromal cells is affected by cell-cell contact with highly metastatic ((LOX)-expressing) or poorly metastatic (LOX-knockdown) MDA-MB-231 cells (1).

Breast cancer cell metastasis depends on interactions with the microenvironment (2). There is a compelling need to dissect how gene transcription in cancer cells changes *in vivo* as they adapt to the microenvironment and to characterize how cancer cells affect transcription in contiguous cells. Current models to study how the tumor microenvironment and cancer cells affect each other are limited because cancer cells must be disaggregated or microdissected from surrounding cells for genetic analysis. This process and subsequent cell sorting or processing can alter gene expression. Immunohistochemical staining is an alternative but renders only a static snapshot of protein expression in tumor tissue. Similarly, microdissection offers a static profile of steady-state transcripts. This proposal modifies a recently-described metabolic labeling technique (3) as a tool for dynamic characterization of tumor cell-microenvironment interactions.

The initial focus is on interactions between tumor cells and bone marrow stroma. No studies to date have examined the effects of breast cancer cells on global gene transcription in bone marrow stroma. It has recently been shown that even a static genetic signature in the stroma of primary breast cancer tumors is prognostic of cancer outcome (4). An underling **hypothesis** of this proposal is that that breast cancer bone metastasis depends upon gene expression cascades that are reinforced by both juxtacrine (cell-cell contact) and paracrine loops between bone marrow stroma and tumor cells. It is also hypothesized that these gene expression cascades depend on the expression of lysyl oxidase that has been shown to be required for sustaining metastasis in a murine model (1).

Our approach to testing this hypothesis has been to transduce human HS-27a bone marrow stromal cells (5) with the toxoplasma gondii enzyme uracil phosphoribosyl transferase (UPRT) constructed as a fusion protein with a C-terminal fluorescent tag (yellow fluorescent protein (YFP)). This enzyme enables eukaryotic cells to incorporate exogenous thiouracil into their RNA (3). RNAs synthesized during a thiouracil pulse are subsequently labeled with biotin and isolated on streptavidin beads. RNA that is concurrently harvested from cocultured breast cancer cells lacks thiol-tags and is therefore not biotinylated or isolated on the beads. Microarray analysis of bead-purified mRNA from mixtures of stromal and cancer cells will therefore specifically detect the stromal cell transcripts that were made during the thiouracil pulse. In order to determine the effects of cancer cell lysyl oxidase on transcription in adacent bone marrow stromal cells, the stromal cells have been cocultured either with MDA-MB-231 cells expressing vector control or LOX knockdown shRNA. These cancer cells are tagged with red fluorescent protein (dsRed2) in order easily to visualize the mixing of the cancer cells on the stromal monolayer. Controls include stromal cells and cancer cells cultured alone. In order to distinguish juxtacrine versus paracrine interactions, another control consists of stromal cells exposed to conditioned medium from the cancer cells. Figure One summarizes our approach.

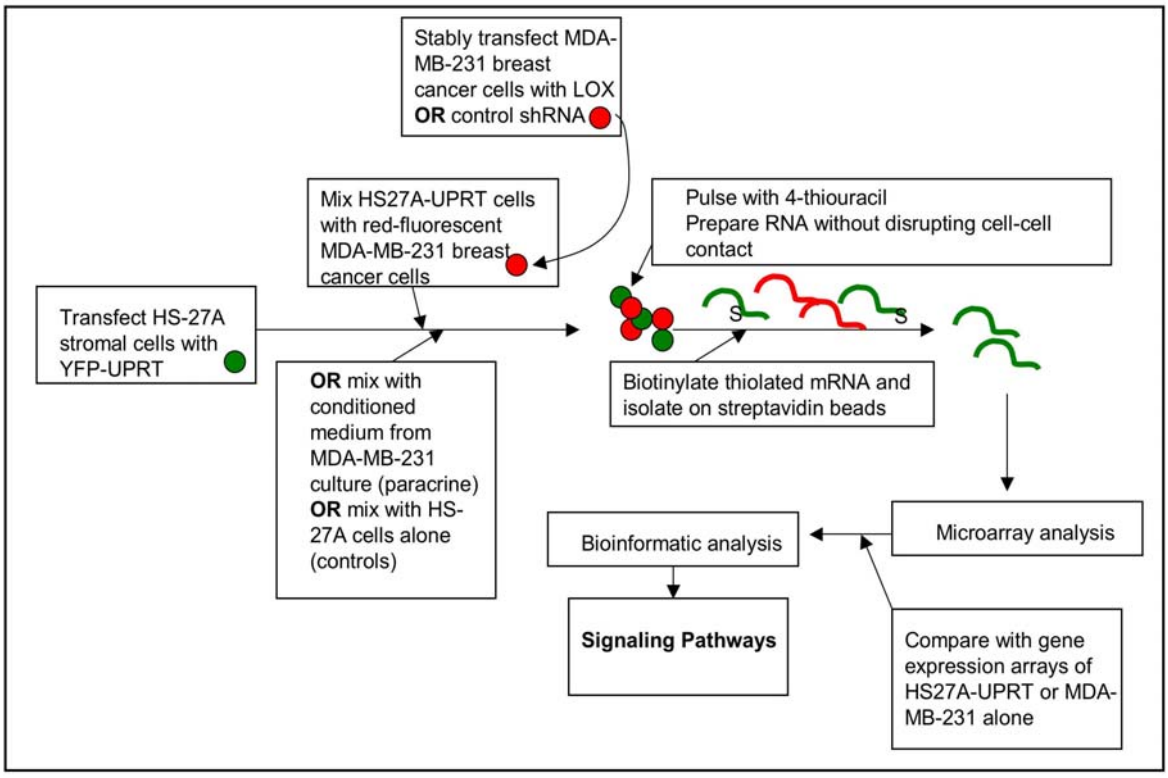

Figure 1. Concept of cell-type specific mRNA isolation using UPRT and thiouracil pulsing. Transcription specific to HS-27A stromal cells occurring during a thiouracil pulse is shown. Stromal transcription arising from juxtacrine interactions between cancer and stromal cells is captured in the approach shown. For paracrine-specific activation, cells would be separated by transwells or conditioned medium from cancer cells is used. In each case, transcription of stromal cells would be compared to that in stromal cells cultured alone.

The goal of this strategy is to identify novel components of a cancer-stroma communication network that are amenable to targeted therapy.

BODY

The following steps are important components of our project execution, as listed in our Statement of Work. We have made significant progress in this project, completing SOW goals #1, 3, 4, 5, 6, 7 and 8. SOW #2 focused on an alternative stromal cell line to use in this work but multiple cultures were nonviable and this alternative was dropped. Work was delayed because of difficulty in generating red fluorescent breast cancer cells in which lysyl oxidase expression was stably suppressed. Initial attempts to do this using interfering RNA were not successful. We have now succeeded in this aim (SOW #4) that is a key component of completing the work. The modified lysyl oxidase knockdown and control cells were then able to be used in mixing experiments with stromal cells (SOW 8) this past month.

Sections below summarize or report our data relative to the SOW.

SOW 1) Establish bone marrow stromal cell lines stably transfected with a YFP-UPRT (uracil phosphoribosyltransferase) fusion protein.

We have successfully transduced and stably expressed a YFP-UPRT transgene in the the HS-27a stromal cell line (5) (Figure 2)

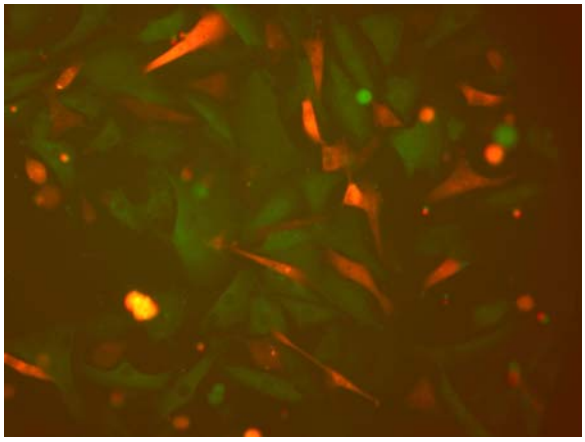

Figure 2. Bone marrow stromal cells stably expressing YFP-UPRT (green) cocultured with MDA-MB-231 breast cancer cells in which LOX has been knocked down and which express red fluorescent protein (red). Mixed distribution at two days is shown.

SOW 2) Compare YFP-UPRT expression in two different bone marrow
stromal cell lines (HS-5 and HS-27A). As noted above, we have chosen to focus on HS-27a cells for technical reasons.

SOW 3) Establish activity of UPRT in the two stably transfected stromal cell lines by streptavidin-HRP blotting of fractionated mRNA obtained after thiouracil pulse of cells. Optimize timing of pulse. Select one cell line for further study.

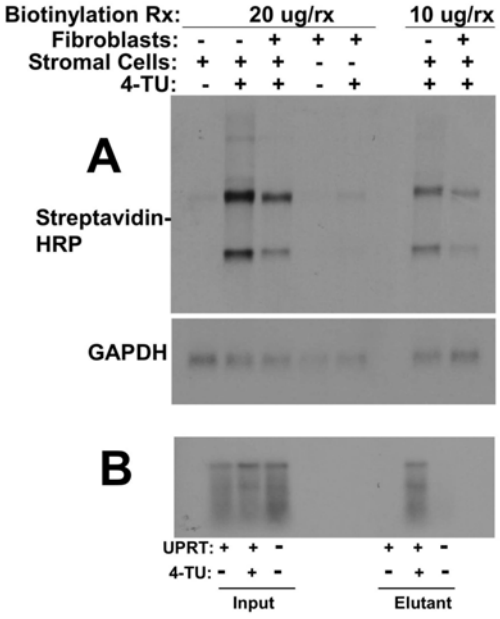

Figure 3. **A. Expression and activity of YFP-UPRT in a stromal cell line**. Top-Streptavidin western blot of YFP-UPRT-expressing HS-27a cells cultured alone or with immortalized primary fibroblasts that do not express UPRT. Cells were incubated for 6 hours either with DMSO vehicle (-) or 4-thiouracil (4-TU) as indicated. RNA from all samples was harvested, biotinylated and blotted with streptavidin-HRP. Biotinylation of 4-TU pulsed sample is evident for HS-27a cells but not for fibroblasts cultured alone Intensity of signal is decreased when half of cells in flask are fibroblasts lacking UPRT. Different biotin-coupling titrations are also shown. Bottom-RNA loading is shown by GAPDH. **B. Specific capture of thiolated, biotinylated mRNA on streptavidin beads.** Radiolabeled input RNA and eluted RNA shown.

SOW 4) Measure lysyl oxidase (LOX) message in control- and in LOX-knockdown MB-MDA-231 cells.

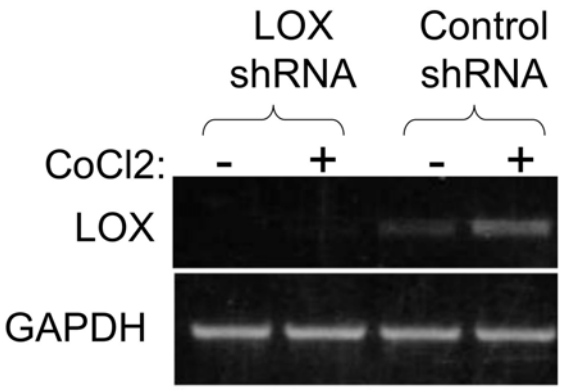

Figure 4. A. LOX knockdown. Stable downmodulation of LOX in MBA-MB-231 breast cancer cells by shRNA is shown. Cells are exposed to the hypoxia mimick cobalt chloride (CoCl) as indicated.

SOW 5) Establish stable red fluorescent protein transfectants of control- and LOX-knockdown MB-MDA-231 cells.
Completed. See figure 2 for example.

SOW 6) Optimize co-culture conditions for UPRT-expressing bone marrow stromal cells and for MB-MDA-231 cells.
We have done this and ultimately decided that establishing a confluent monolayer of stromal cells upon which an equal or twice equal number of breast cancer cells are plated for 48 hours offers significant interaction time. The cancer cells bind to the stromal cells in this interval and their mRNA cannot be biotinylated, whereas the stromal cell mRNA can be.

See also Figure 2 above.

SOW 7) Optimize isolation of thiouracil pulsed mRNA.
After extensive testing, we have found that MPG streptavidin beads and M-280 streptavidin dynabeads are optimal for thiolated-biotinylated mRNA isolation. We have optimized bead to biotinylated mRNA at 1 ul (5 mg/ml) beads per 5 ug of UPRT mRNA, reaction volume at 4X the bead volume and wash volume at 12X the bead volume; protocol is otherwise as in reference 3.

SOW 8) Isolate thiouracil-pulsed mRNA from cocultured cells.
See Figure 3 and figure 5 below.

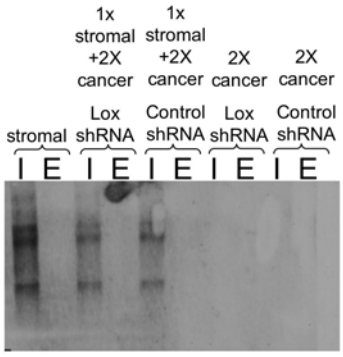

Figure 5. Example of thiouracil-incorporation in HS-27A stromal cells cocultured with MDA-MB-231 breast cancer cells. A streptavidin-HRP blot of 2 ug input (I) mRNA and of mRNA eluted from beads (E) is shown. Note that stromal cells give a strong input signal and that the signal is decreased by ~60% when stromal cell input mRNA is diluted with mRNA from lox- or control-knockdown breast cancer cells. The mRNA from the breast cancer cells does not incorporate thiouracil from the 4-thiouracil pulse as shown by lack of signal in lanes representing input from cancer cells alone. No signal is evident in any of the eluted mRNAs because biotin is cleaved during the elution process.

SOW 9) Biotinylate thiouracil-pulsed mRNA and isolate it on streptavidin beads.
See Figure 5 above. Figure 6 Northern blot (above) shows presence of eluted GAPDH + Actin detectable in elutants from HS27a stromal cells incubated with 4-thiouracil (4-TU) but not with DMSO vehicle. A range of exposures showed lack of message in DMSO elutants and presence in 4-TU elutants (not shown). Streptavidin western blotting showed that only the cells exposed to 4-TU could be biotinylated and that ~90% of the biotinylated mRNA was
captured by the beads and absent in the flowthrough.

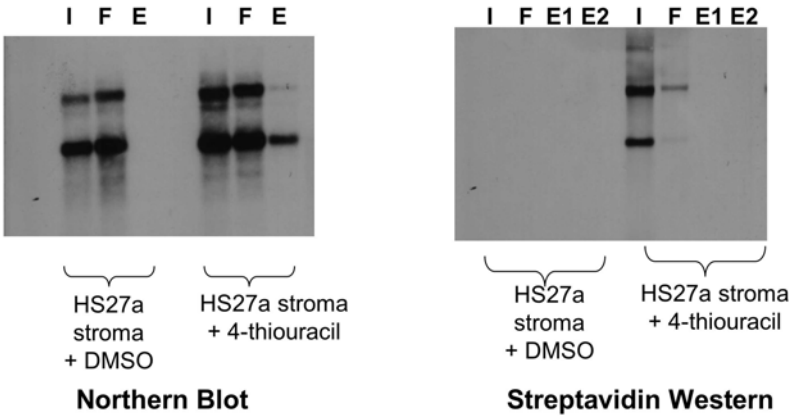

Figure 6. Elution of message from stromal cells incubated with thiouracil (but not with vehicle) as shown on Northern blotting with actin + GAPDH. Streptavidin blotting shows biotinylation only of RNA from stromal cells incubatted with thiouracil. ~90% binds to beads. Input (I), flowthrough (F), elutant (E; E1 and E2 are 2 sequential elutants) are indicated.

SOW 10) Use isolated biotinylated mRNA to probe expression microarrays.
We are scaling up in order to do this during a no-cost extension year

SOW 11) Analyze results.
We will conduct bioinformatic analyses and validation during a no-cost extension year.

KEY RESEARCH ACCOMPLISHMENTS
•Specific thiol-labeling of stromal cell mRNA within a heterogeneous cell mixture can be achieved using a YFP-UPRT transgene.

•Stable downmodulation of lysyl oxidase mRNA in MDA-MB-231 breast cancer cells.

•Demonstration of functionality of a YFP-UPRT transgene.

•Tagged stromal cell mRNA can be efficiently recovered from a mixture of RNAs from different cell types.

REPORTABLE OUTCOMES
Poster presentation at the 2008 Era of Hope Meeting, Baltimore, MD, June 25-28, 2008.
Richard Steinman, “The Dialogue of Metastasis-Uncovering Juxtacrine Genetic Cascades with a Toxoplasma Gondii Enzyme.”

CONCLUSIONS

Preliminary results support the feasibility of measuring molecular communication between breast cancer cells and bone marrow stromal cells at the level of cell-specific pulsed transcription for the first time. This strategy could identify novel components of a cancer-stroma communication network that are amenable to targeted therapy.

The technique used in this proposal features unprecedented real-time dynamic characterization of bone marrow stromal cell-*specific* transcription without disrupting cell-cell contacts. Despite theimportance of cancer cell-stromal interactions, current approaches are poorly suited toanalyze the effect of cancer cells on their microenvironment (and vice versa) within intact tumors. By laying the groundwork for such *in vivo* analysis, these investigations should make it feasible for the first time to kinetically profile transcription that arises from cell-cell contacts in distinct subsets of cells within tumors.

The ability to specifically label stromal cell mRNA during a pulse could be adapted to examine stromal cell transcription in different time windows after contact with breast cancer cells, or in the context cancer cell or stromal cell exposure to drugs. Lastly, we have prepared all necessary reagents to test whether lysyl oxidase sup[ports metastatic aggressiveness of breast cancer cells by enabling them to durably alter the transcription in neighboring bone marrow stromal cells in ways that less aggressive breast cancer cells do not.

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